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Xyloside Transport by XylP, a Member of the Galactoside-Pentoside-Hexuronide Family*[§]

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This paper describes the functional characterization of the xyloside transporter, XylP, of *Lactobacillus pentosus* with the aid of a spectroscopy-based assay system. In order to monitor the transport reaction, the natural xyloside isoprimeverose, a building block of hemicellulose, and the analogue methyl-isoprimeverose were chemically synthesized by a new and efficient procedure. The XylP protein was purified by metal affinity chromatography, following high level expression in *Lactococcus lactis* from the nisin-inducible promoter. The purified XylP protein was incorporated into liposomes, in which the glucose dehydrogenase from *Acinetobacter calcoaceticus* (sGDH) was entrapped. sGDH can oxidize aldose sugars in the presence of dichlorophenol-indophenol as electron acceptor. The coupled assay thus involves XylP-mediated isoprimeverose uptake followed by internal oxidation of the sugar by sGDH, which can be monitored from the reduction of 2,6-dichlorophenol-indophenol at 600 nm. The uptake of isoprimeverose was stimulated by the presence of the non-oxidizable methyl-isoprimeverose on the *trans*-side of the membrane, indicating that exchange transport is faster than unidirectional downhill uptake. Unlike other members of the galactoside-pentoside-hexuronide family, XylP does not transport monosaccharides (xylose) but requires a glycosidic linkage at the anomeric carbon position. Consistent with a proton motive force-driven mechanism, the uptake was stimulated by a membrane potential (inside negative relative to outside) and inhibited by a pH gradient (inside acidic relative to outside). The advantages of the here-described transport assay for studies of carbohydrate transport are discussed.

The XylP protein of *Lactobacillus pentosus* is a secondary transport system belonging to the galactoside-pentoside-hexuronide transporter (GPH)¹ family (1). The gene encoding the

XylP protein is clustered with those of a xylose isomerase (*xylA*), a xylulose kinase (*xylB*), a regulator protein (*xylR*), and a xylosidase (*xylQ*) (2–5). Since the *xylP* gene forms part of the *xyl* operon and is induced in the presence of xylose, the XylP protein was initially thought to be involved in the transport of xylose (4). The discovery that another gene (*xylQ*) of the *xyl* operon encoded an intracellular xylosidase provided first evidence that XylP is a xyloside transporter (6). In many microorganisms, homologues of the *xylP* and *xylQ* genes are clustered together and the corresponding proteins are likely to have a role in the uptake and metabolism of xylosides. Xylosides form the building blocks of different hemicelluloses, which are sugar polymers in the cell walls of plants, e.g. xylobiose in xylans and isoprimeverose in xyloglucans. Although abundant in nature, the fate of these disaccharides in bacterial metabolism is poorly documented.

Isoprimeverose is a disaccharide of xylose α -1,6 linked to glucose (Fig. 1). It is not readily isolated from cell wall material since the enzymatic degradation of xyloglucan is inefficient due to its low solubility in water, and the separation of the different degradation products is difficult. Helferich and Rauch first reported the synthesis of primeverose in 1927 (7). Later, Zemplén and Bognár reported a modified procedure for the preparation of primeverose and isoprimeverose (8). In this paper a new and more efficient procedure for the synthesis of large amounts of isoprimeverose and methyl-isoprimeverose (Fig. 1) is described.

Despite the successful synthesis of isoprimeverose, this non-radioactive substrate could not be used in the conventional transport assays. This inspired us to invent a spectroscopic assay for the analysis of carbohydrate transport reactions without the need of radiolabeled substrates (9). The method makes use of a membrane system bearing the sugar transporter of interest and containing a PQQ-dependent carbohydrate dehydrogenase (sGDH) internally. sGDH, originating from *Acinetobacter calcoaceticus*, has a high affinity for glucose but the enzyme also oxidizes a wide variety of mono- and disaccharides, including isoprimeverose and xylobiose, in the presence of the artificial electron acceptor dichlorophenol-indophenol (Cl₂Ind) (10–12). Cl₂Ind has a high extinction coefficient with absorption maximum at 600 nm, which enables one to follow its reduction spectrophotometrically with high sensitivity even in the presence of membranes. The method had to be modified in order to decrease the sGDH background activity.

In this study, we report on the amplified expression and purification of XylP, the membrane reconstitution of the transporter, and the functional analysis of XylP-mediated translocation by a spectroscopy-based transport assay.

EXPERIMENTAL PROCEDURES

Materials—M17 broth was obtained from Difco. The sGDH and PQQ were a gift from Prof. J. A. Duine (Technical University of Delft, Delft, The Netherlands). Nickel-nitrilotriacetic acid resin was obtained from

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains a description of the synthesis of isoprimeverose and methyl- α -D-isoprimeverose.

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¹ The abbreviations used are: GPH, galactoside-pentoside-hexuronide transporter; Cl₂Ind, 2,6-dichlorophenol-indophenol; DDM, *n*-dodecyl- β -D-maltoside; sGDH, soluble quinoprotein glucose dehydrogenase; isoprimeverose, α -D-xylopyranosyl-1,6- α -D-glucopyranoside; OG, *n*-octyl- β -D-glucopyranoside; PQQ, pyrroloquinoline quinone; MOPS, 4-morpholinepropanesulfonic acid; diSC₃(5),3,3'-dipropylthiadicarbocyanine iodide.

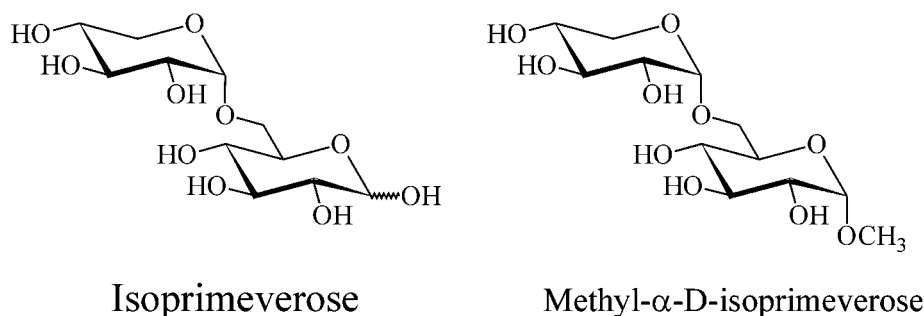
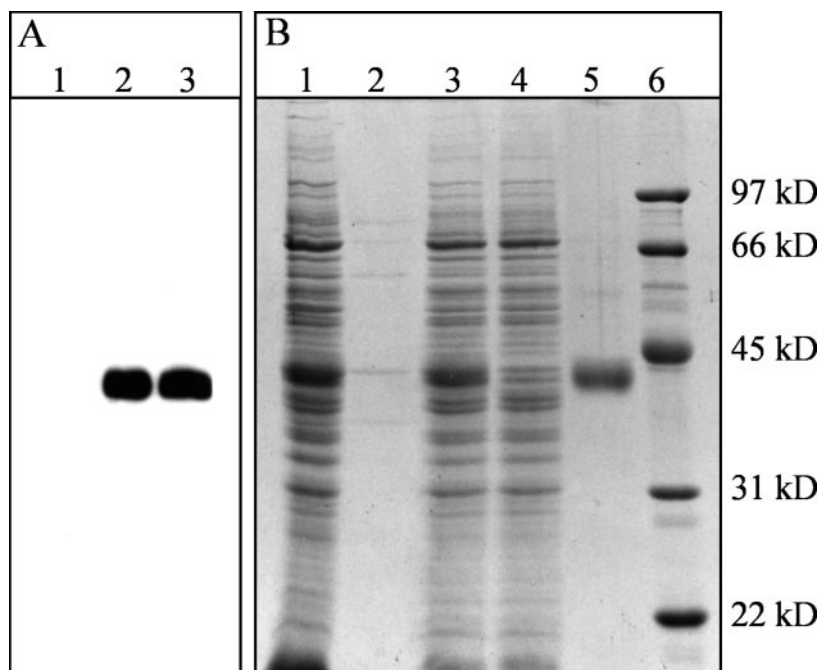


FIG. 1. Structures of isoprimeverose and methyl-α-D-isoprimeverose.

FIG. 2. Amplified expression and purification of the XylP protein. A, Western blot of inside-out membrane vesicles of *L. lactis* NZ9000/pNZ8048xylP. At different time points, cells were collected by centrifugation. Cells were disrupted by sonication (15 s on, 45 s off, 3 cycles, amplitude of 4 μ m) and boiling for 5 min in sample buffer containing 2% SDS prior to SDS-polyacrylamide gel electrophoresis. Lane 1, before induction; lane 2, 1-h induction with nisin; lane 3, 2-h induction with nisin; 10 μ g of protein was loaded in each lane. B, Coomassie Brilliant Blue-stained SDS-polyacrylamide (10%) gel. Lane 1, inside-out membrane vesicles of *L. lactis* NZ9000/pNZ8048xylP (20 μ g of protein); lane 2, pellet fraction after solubilization with 1% DDM; lane 3, solubilize; lane 4, flow-through; lane 5, elution with 200 mM imidazole (5 μ g of protein); lane 6, molecular weight marker.



Qiagen, Inc., Biobeads SM-2 from Bio-Rad, *n*-dodecyl- β -D-maltoside from Anatrace, Triton X-100 and *n*-octyl- β -D-glucopyranoside from Roche Molecular Biochemicals. Total *Escherichia coli* lipids were obtained from Avanti Polar Lipids, and L- α -phosphatidylcholine from egg yolk was from Sigma. The synthesis of isoprimeverose and methyl- α -D-isoprimeverose is described in the on-line supplement to this report (32). All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Growth Conditions—*Lactococcus lactis* NZ9000 (13) was cultivated semi-anaerobically at 30 °C in M17 broth, pH 6.6, supplemented with 0.5% (w/v) glucose and 5 μ g/ml chloramphenicol when carrying the plasmid pNZ8048xylP. For the isolation of membranes, *L. lactis* NZ9000/pNZ8048xylP were cultivated in a 10-liter pH-regulated fermentor to an A_{600} of 3, after which transcription from the nisin promoter was switched on by the addition of 0.4% (v/v) culture supernatant of the nisin-producing strain NZ9700 (14). The final concentration of nisin was about 4 ng/ml. After 1 h of induction, cells were harvested and inside-out membrane vesicles were prepared.

Plasmid Construction—For the cloning of the *xylP* gene into the nisin inducible expression system of *L. lactis*, the plasmid pLPA1 (6) was used as a template to amplify the *xylP* gene using the oligonucleotides 5'-CATCGATGTTGGATTTCATGAGCGTTAGTA-3' and 5'-CGCGGATCCCTTTTGATCGTCA-3', thereby engineering a *Bsp*HI restriction site at the start and a *Bam*HI site at the stop codon of the gene. These restriction sites were used to replace the *oppA* gene in the plasmid pNZ8048oppah (15) for the *xylP* gene. Herewith, the *xylP* gene was placed in frame with a sequence (already present in the pNZ8048oppah plasmid) that specifies a 6-histidine tag at the C terminus of the XylP protein. The resulting plasmid, pNZ8048xylP, was transformed into *L. lactis* NZ9000.

Isolation of Membrane Vesicles—For the isolation of inside-out membrane vesicles of *L. lactis*, cells (0.5 g/ml protein) were lysed by passage

through a LAB 2000 homogenizer (Kindler Maschinen AG, Zürich, Switzerland) (15,000 p.s.i.), following (partial) digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30 °C (16). The membrane preparations were stored in liquid nitrogen. The protein concentration of the membranes was determined with the Bio-Rad DC Protein Assay according to their instructions (Bio-Rad).

Immunoblotting—The relative concentrations of XylP protein were determined by immunodetection with antibodies raised against the 6-histidine tag (Dianova GmbH). The proteins were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using Western-Light[®] chemiluminescence detection kit with CSPD[®] as a substrate, was performed as recommended by the manufacturer (Tropic Inc.).

Purification, Stability, and Membrane Reconstitution of XylP—The solubilization and purification of the XylP protein was performed as described (17) with the following modifications; membranes were solubilized in buffer A (50 mM potassium phosphate, pH 8.0, 10 mM imidazole, 100 mM NaCl, 10% (v/v) glycerol) supplemented with 1% *n*-dodecyl- β -D-maltoside (DDM) (w/v). The column was washed successively with 20 column volumes of buffer A containing 0.05% DDM and 10 column volumes of buffer A containing 25 mM imidazole, pH 8.0, and 0.05% DDM. XylP was eluted from the column in buffer B (50 mM potassium phosphate, pH 7.0, 200 mM imidazole, 100 mM NaCl, and 10% (v/v) glycerol) plus 0.05% DDM. For determination of the stability of XylP in detergent solution, the protein was dialyzed overnight against buffers of different composition. When the effect of detergent on protein stability was tested, DDM was replaced on the column after the second wash step by equilibrating the nitrilotriacetic acid resin with 10 column volumes buffer A and eluting XylP in buffer B, both containing the appropriate detergent. To determine the degree of aggregation of XylP in detergent-solution, protein spectra were taken with a Carry 100

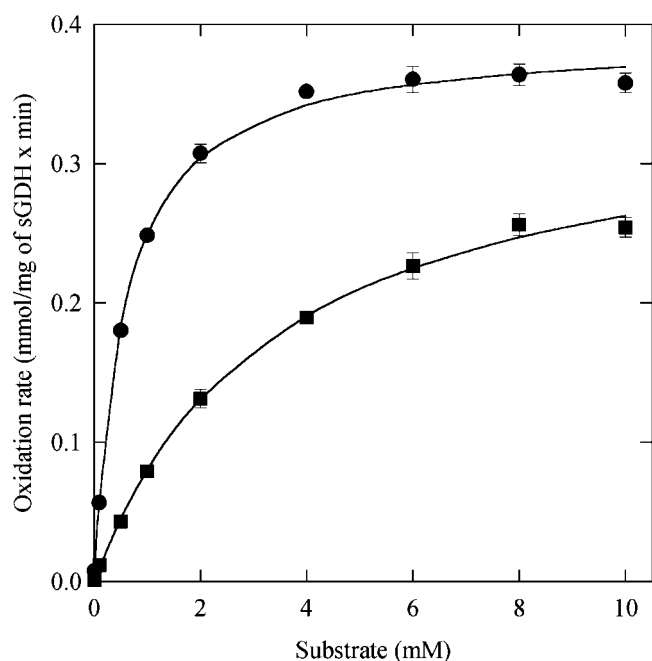


FIG. 3. **Kinetics of carbohydrate oxidation by sGDH.** The assays were performed in 50 mM potassium phosphate, pH 7.0, supplemented with 50 μ M Cl_2Ind and 78 ng/ml sGDH. The reaction was started by the addition of various concentrations of isoprimeverose (■) or maltose (●). The data were fitted to the Michaelis-Menten equation. The error bars based on three independent measurements are indicated.

Bio UV-visible spectrophotometer (Varian) from 240 to 340 nm. The concentration of purified XylP was determined by measurement of the absorption at 280 nm ($\epsilon_{280} = 100,800 \text{ M}^{-1} \text{ cm}^{-1}$).

For membrane reconstitution of XylP by detergent dilution, DDM was replaced by *n*-octyl- β -D-glucoside (OG) by washing the column with 10 column volumes of buffer C (50 mM potassium phosphate buffer, pH 8, 10 mM imidazole, pH 8.0, 100 mM NaCl, 20% glycerol, and 1.25% OG (w/v)). The protein was eluted from the column in buffer C at pH 7.0, containing 200 mM imidazole. After purification, 2 ml of purified XylP protein (0.1 mg/ml) was mixed with 400 μ l of liposomes (20 mg/ml) resulting in a 1:40 protein to lipid ratio (w/w). This mixture was incubated on ice for 10 min, after which it was diluted rapidly into 80 ml of 50 mM potassium phosphate, pH 7.0. The proteoliposomes were collected by centrifugation (3 h, $180,000 \times g$, 4 °C) and stored in liquid nitrogen with or without sGDH (see below) until use. Membrane reconstitution protocols involving Triton X-100- or DDM-stabilized liposomes and detergent removal by adsorption onto polystyrene beads (BioBeads) were in essence similar to those described previously for the lactose transporter of *Streptococcus thermophilus* (17, 18). Liposomes were prepared from acetone/ether washed *E. coli* lipids and L- α -phosphatidylcholine from egg yolk in a ratio of 3:1 (w/w), and dissolved in 50 mM potassium phosphate, pH 7.0, to a final phospholipid concentration of 20 mg/ml.

Freeze-fracture Electron Microscopy—For the preparation of freeze-fracture replicas, XylP proteoliposomes went through two cycles of freeze-thawing in order to generate large vesicle structures. The replicas were generated as described (19). For particle density measurements, images were enlarged at a final magnification of $\times 250,000$, and intramembrane particles from both convex and concave proteoliposomes were counted from known areas of the membrane.

sGDH Reconstitution and Activity Assay—sGDH was prepared as described (20), using a 1.5-fold excess of PQQ over apo-sGDH. Briefly, sGDH and PQQ were mixed at final concentrations of 25 and 37.5 μ M, respectively, in 20 mM MOPS, pH 7.0, plus 3 mM CaCl_2 . The mixture was incubated for 15 min at room temperature and, subsequently, used in the enzymatic assays or for enclosure in proteoliposomes. Enzyme assays were performed in 50 mM potassium phosphate, pH 7.0, supplemented with 50 μ M Cl_2Ind (2,6-dichlorophenol-indophenol) and varying concentrations of substrate. The reactions were started by the addition of 78 ng/ml sGDH (enzyme samples were diluted with 20 mM MOPS, pH 7.0, 3 mM CaCl_2 , and 0.1% Triton X-100), and the enzyme activity was determined spectrophotometrically at 25 °C by following the reduction of Cl_2Ind at 600 nm (20). All measurements were performed on a Carry

100 Bio UV-visible spectrophotometer (Varian) spectrophotometer.

Enclosure of sGDH in Proteoliposomes—sGDH was enclosed in XylP proteoliposomes as described (9), with some modifications. The concentration of enclosed sGDH was 65 μ g/ml, unless indicated otherwise. To remove external sGDH, the proteoliposomes were washed by centrifugation (15 min, $185,000 \times g$, 10 °C) using 3 ml of 50 mM potassium phosphate, pH 7.0, in the first wash and 3 ml of 50 mM potassium phosphate, pH 8.0, containing 4 mM EDTA, in the second wash. The proteoliposomes were resuspended in 400 μ l of 50 mM potassium phosphate, pH 8.0, supplemented with 4 mM EDTA and incubated for 30 min at 50 °C. After 30 min of incubation, they were washed two times by centrifugation with 50 mM potassium phosphate, pH 7.0, plus 4 mM EDTA. Finally, the proteoliposomes were resuspended in 50 mM potassium phosphate, pH 7.0, supplemented with 4 mM EDTA, to a final concentration of 2 mg/ml XylP, which corresponds to 80 mg/ml phospholipid. When proteoliposomes were used for counterflow-type experiments, the membranes were resuspended in 50 mM potassium phosphate, pH 7.0, supplemented with 25 mM methyl-isoprimeverose and incubated overnight. To impose an artificial membrane potential, the enclosure of sGDH was performed in buffer D (100 mM potassium acetate, pH 7.0, 20 mM potassium phosphate, pH 7.0, and 1 mM MgSO_4) or in buffer E (50 mM potassium acetate, pH 7.0, 50 mM methylamine, pH 7.0, 20 mM potassium phosphate, pH 7.0, and 1 mM MgSO_4). The proteoliposomes were washed and resuspended in these buffers supplemented with 4 mM EDTA. The final protein concentration was 2 mg/ml XylP.

Spectrophotometric Measurements of XylP Transport Activity—Transport of sugars was determined from the decrease in absorption of Cl_2Ind at 600 nm and at a temperature of 25 °C ($\epsilon_{600} = 20.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture consisted of 50 mM potassium phosphate, pH 7.0, supplemented with 50 μ M Cl_2Ind and varying concentrations of sugar, unless indicated otherwise. The reaction was started by the addition of sGDH-containing proteoliposomes. A membrane potential was generated by diluting proteoliposomes, which were prepared in buffer D or E plus 4 mM EDTA, into buffer F (100 mM sodium acetate, pH 7.0, 20 mM sodium phosphate, pH 7.0, 1 mM MgSO_4) or buffer G (50 mM sodium acetate, pH 7.0, 50 mM methylamine, pH 7.0, 20 mM sodium phosphate, pH 7.0, 1 mM MgSO_4), respectively. For analysis of the data, the assumption was made that the α - and β -anomer of isoprimeverose are present in solution in a 2:3 ratio. Because sGDH is β -anomer-specific and the spontaneous mutarotation is slow compared with the transport rates, oxidation rates were multiplied by 1.66 to obtain the actual transport rates.

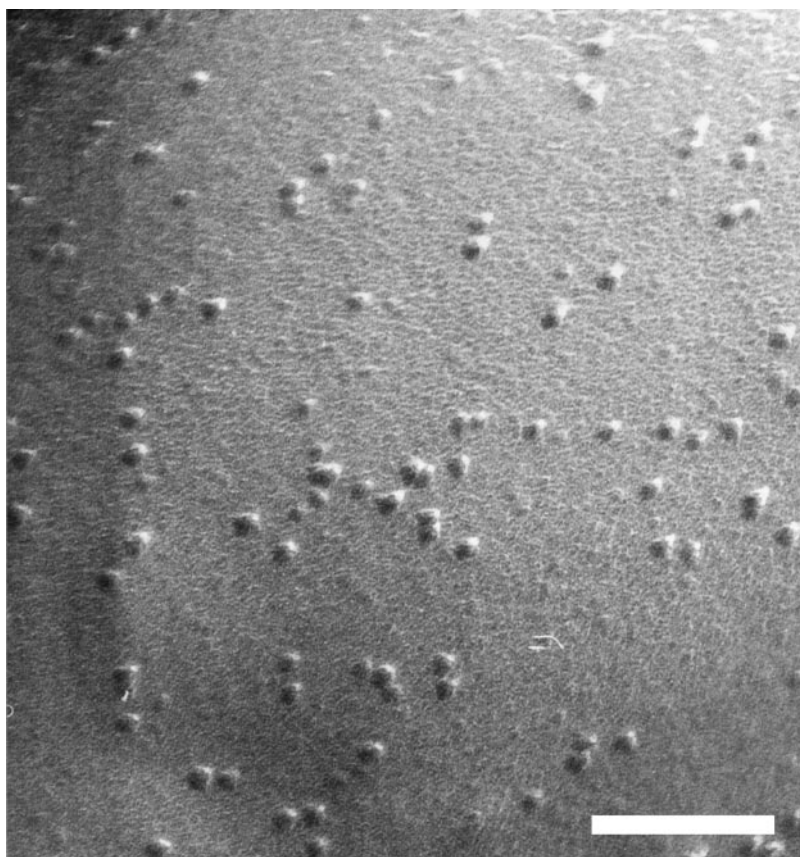
Measurements of $\Delta\psi$ and ΔpH —The magnitude of the membrane potential ($\Delta\psi$) and ΔpH were analyzed by fluorescence measurements, using diSC₃(5) and pyranine as probes, respectively. For the $\Delta\psi$ measurements, 2 μ l of proteoliposomes (2 mg/ml XylP protein and 80 mg/ml lipids) were diluted into 800 μ l of buffer D, E, F, or G containing 1.25 μ M diSC₃(5). The $\Delta\psi$ was generated by the addition of 0.25 μ M valinomycin from a 200 μ M stock solution in ethanol. The membrane potential was dissipated after 3 min by the addition of 0.125 μ M nigericin from a 100 μ M stock solution in ethanol. The final ethanol concentration never exceeded 0.5%. The excitation and emission wavelengths used for the diSC₃(5) measurements were 643 and 666 nm, respectively. The changes in ΔpH , following the generation of a $\Delta\psi$, were measured using proteoliposomes that were preloaded with 0.5 mM pyranine; 4.8 μ l of proteoliposomes (2 mg/ml XylP protein and 80 mg/ml lipids) were diluted into 800 μ l of buffer D, E, F, or G. The excitation and emission wavelengths used for the pyranine measurements were 461 and 511 nm, respectively. All measurements were performed at a constant temperature of 25 °C.

RESULTS

Overexpression of XylP in *L. lactis*—The nisin-inducible expression system of *L. lactis* was used for the amplification of XylP (13). The *xylP* gene was placed under control of the strong and tight *nisA* promoter. Immunoblots showed that XylP was not detectable in the absence of the inducer, whereas maximal amplification was obtained after 1 h of incubation with 4 ng/ml nisin (Fig. 2A). The level of expression was $\sim 10\%$ of total membrane protein. Like most other integral membrane proteins, XylP migrated at a lower molecular mass than predicted from the deduced amino acid sequence, i.e. ~ 42 instead of 54 kDa (including the 6-histidine tag).

Solubilization, Purification, and Stability of XylP—As starting material for the solubilization and purification of XylP,

FIG. 4. Freeze-fracture electron micrograph of membrane-reconstituted XylP. XylP was reconstituted at a protein to lipid ratio of 1:40 (w/w) using the *n*-octyl- β -D-glucoside dilution-based method. The image was enlarged (final magnification, $\times 250,000$) (*bar* = 100 nm). For particle density measurements, 1994 particles were counted from a known area.



inside-out membrane vesicles were prepared from *L. lactis* NZ9000/pNZ8048xylP. Various detergents at different concentrations were tested for the solubilization of XylP, of which DDM turned out to be the most effective. Nearly all XylP protein was present in the solubilizate when DDM was present at a final concentration of 1% (w/v) and at a membrane vesicle concentration of 4 mg/ml (Fig. 2B, lanes 2 and 3). The His-tagged protein was purified to near homogeneity in a single step, using nickel affinity chromatography. Most contaminants were removed by washing the column with 20 column volumes of 10 mM imidazole wash buffer, pH 8. The remaining minor impurities were removed by washing the column with 10 column volumes of 25 mM imidazole wash buffer, pH 8.0. The protein was eluted from the column by raising the imidazole concentration to 200 mM and lowering of the pH to 7.0 (Fig. 2B, lane 5). The stability of purified XylP in detergent solution was studied spectroscopically by analyzing the protein spectrum in the 310–340-nm region, *i.e.* a flat line in this region indicates that higher order aggregates are not present. Of the various detergents tested, *n*-dodecyl- β -D-maltoside proved to be superior in terms of extent of solubilization of the membrane vesicles and retention of the native structure of the purified XylP protein. The optimization of the isolation/purification procedure also included the systematic variation of pH, lipid concentration, and glycerol. Under optimal conditions, *i.e.* 50 mM potassium phosphate, pH 7.0, 10% glycerol, and 100 mM NaCl, the protein was stable for at least 4 days at 4 °C. Although the highest stability was achieved in DDM, for reconstitution experiments *n*-octyl- β -D-glucoside had to be used as will be described below. It was observed that XylP was stable in this detergent for at least a few hours, which is within the period of time needed for the membrane reconstitution.

Specificity of sGDH for Isoprimeverose and Methyl-isoprimeverose—The synthesis and characterization of isoprimeverose and methyl- α -D-isoprimeverose is described in the

on-line supplement. sGDH oxidizes a wide variety of mono- and disaccharides, including the disaccharide isoprimeverose. sGDH has an absolute preference for the β -anomer of aldoses (21). Since the disaccharide maltose is not a substrate of XylP, it was used to obtain an estimate of sGDH associated with the outer surface of the membranes. In order to be able to make the appropriate corrections for isoprimeverose oxidation not associated with XylP-mediated transport, the kinetics of oxidation of isoprimeverose and maltose was determined first (Fig. 3). The apparent K_m and V_{max} of sGDH for isoprimeverose and maltose were 3 ± 0.3 mM and 345 ± 20 $\mu\text{mol mg}^{-1} \text{min}^{-1}$, and 0.57 ± 0.03 mM and 383 ± 4 $\mu\text{mol mg}^{-1} \text{min}^{-1}$, respectively; the \pm values reflect the standard error of the experiments. Importantly, when the anomeric carbon of isoprimeverose was methylated, the sugar could no longer be oxidized.

Thermal Inactivation of External sGDH—Due to its high isoelectric point of 9.5, sGDH has the tendency to tightly associate with membranes containing anionic lipids. Since the turnover of sGDH is at least 2 orders of magnitude higher than that of XylP, even a small amount of externally located sGDH will give rise to high background activity (9). We succeeded in lowering the external sGDH activity by dissociating Ca^{2+} from sGDH in the presence of EDTA and at temperatures above 35 °C (22). Since EDTA is membrane-impermeable, the internal Ca^{2+} was not complexed, and internal sGDH was reconstituted and fully active again after the temperature was lowered to 25 °C. Control experiments indicated that the membrane-embedded transporter was not affected by the heat treatment.

Isoprimeverose Transport in XylP Proteoliposomes—Although XylP was most stable in DDM, the functional membrane reconstitution of XylP from DDM or Triton X-100 extracts turned out to be problematic. In our hands, this methodology, involving preformed liposomes and polystyrene beads (Biobeads) for detergent removal, is generally superior over detergent (OG) dilution- or dialysis-based reconstitution

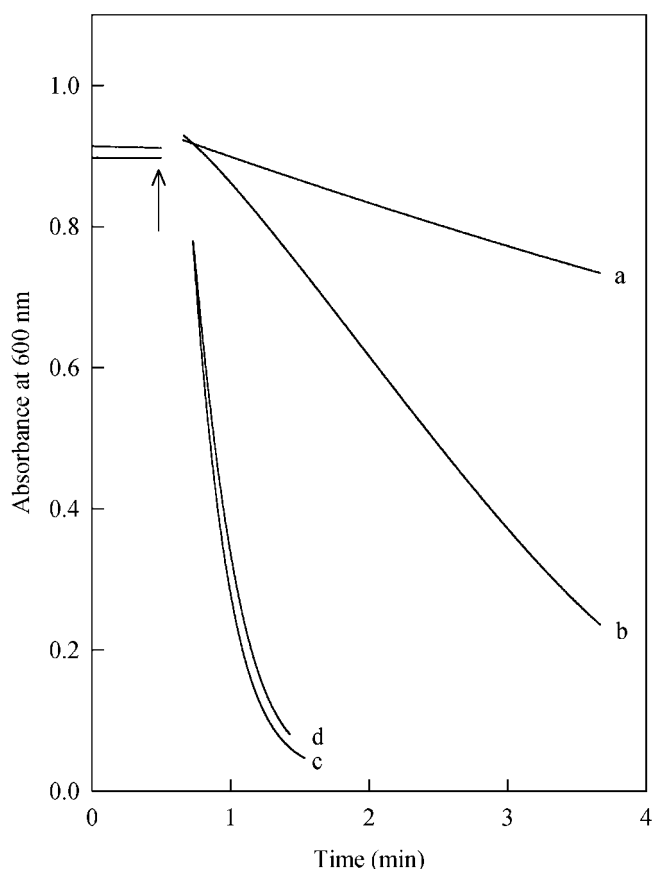


FIG. 5. **Isoprimeverose transport by sGDH-containing XylP proteoliposomes.** The assays were performed in 500 μ l of 50 mM potassium phosphate, pH 7.0, supplemented with 50 μ M Cl_2Ind and the following substrates: a, 5 mM maltose; b, 12.5 mM isoprimeverose; c, 5 mM maltose + 0.1% Triton X-100; d, 12.5 mM isoprimeverose + 0.1% Triton X-100. The arrow indicates the addition of 5 μ l of XylP proteoliposomes (final concentration: 20 μ g/ml XylP).

procedures (17, 18). Analysis of the proteoliposomes by freeze-fracture electron microscopy revealed a very low protein density and a large size distribution when DDM or Triton X-100 was used in combination with Biobeads (data not shown). The failure of this reconstitution procedure most probably lies in the tendency of XylP to form higher oligomeric structures upon slow removal of detergent. Membrane reconstitution based on *n*-octyl- β -D-glucoside dilution is much faster and proved to be more successful. The particle density increased about 5 times, and the particle size was much more homogeneous than with DDM- or Triton X-100-mediated reconstitution into preformed liposomes (Fig. 4). For the analysis of the particle density, 1994 intramembrane particles were counted in 24 different proteoliposomes, resulting in a density of 160 ± 10 particles/ μm^2 . This corresponds to a reconstitution efficiency of $\sim 50\%$, when using a value of 0.6 nm^2 for the surface area of the lipid molecule and an initial protein to lipid ratio of 1:40 (w/w). For this calculation, the assumption was made that freeze-fracturing yields protein particles in either of the two lipid monolayers.

The transport of isoprimeverose by the XylP protein was assayed in the presence of 50 μ M electron acceptor Cl_2Ind . Since Cl_2Ind is freely membrane-permeable, it was not necessary to preload the proteoliposomes with this compound. Upon addition of isoprimeverose, the absorbance at 600 nm decreased immediately (Fig. 5, trace b). To demonstrate that the decrease in absorbance required isoprimeverose transport, maltose was used as a control substrate (Fig. 5, trace a). Since

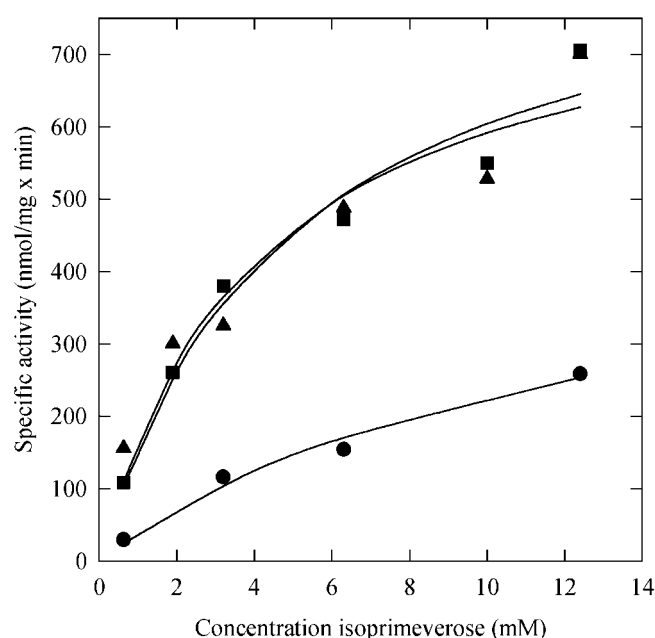


FIG. 6. **Kinetics of isoprimeverose transport by XylP proteoliposomes.** The assays were performed in 500 μ l of potassium phosphate, pH 7.0, supplemented with 50 μ M Cl_2Ind and various concentrations of isoprimeverose. The reactions were started by the addition of 5 μ l of XylP proteoliposomes (final concentration: 20 μ g/ml XylP) preloaded with various concentrations of sGDH: \bullet , 16.25 μ g/ml; \blacksquare , 65 μ g/ml; \blacktriangle , 130 μ g/ml. The data were fitted to the Michaelis-Menten equation. The rates of isoprimeverose transport were corrected for background sGDH activity, using the rates of maltose oxidation and taking into account the differences in V_{max} and K_m values of sGDH for isoprimeverose and maltose.

the affinity of sGDH for maltose is about 5 times higher than for isoprimeverose, a maltose concentration of 5 mM was used instead of 12.5 mM for isoprimeverose. Although at these concentrations the oxidation rates for both substrates are approximately the same with free sGDH, following the transport of the substrate into the proteoliposomes the oxidation of isoprimeverose greatly exceeded that of maltose. The data clearly indicate that the XylP protein has been functionally reconstituted and that transport of xylosides can be measured with this assay. Traces c and d in Fig. 5 demonstrate that at the used substrate concentrations of 5 mM maltose and 12.5 mM isoprimeverose, the sGDH activity was almost equal when the internalized sGDH was released. Measurements were highly reproducible. From six independent experiments, the transport rate in the presence of 3.2 mM isoprimeverose was 420 ± 22 $\text{nmol mg}^{-1} \text{min}^{-1}$.

For the kinetic analysis of the transport reaction, it was important to establish that the transport reaction and not the isoprimeverose oxidation is rate-determining. Kinetic analysis of XylP-mediated uptake in proteoliposomes preloaded with sGDH concentrations ranging from 16.25 to 130 μ g/ml demonstrated that the K_m and V_{max} values did not change any further above 65 μ g/ml sGDH (Fig. 6). At saturating sGDH concentration, the apparent K_m and V_{max} for isoprimeverose transport were 4.5 ± 0.5 mM and 870 ± 45 $\text{nmol mg}^{-1} \text{min}^{-1}$, respectively. The presence or absence of Na^+ ions did not affect the rate of transport of isoprimeverose (data not shown).

XylP-mediated Xyloside Exchange—Other members of the GPH family are capable of catalyzing an exchange reaction of two substrates in opposite direction. This mode of transport is faster than the proton-symport reaction in case of the LacS protein of *S. thermophilus*. The chemical synthesis of methyl-isoprimeverose enabled us to investigate the influence of inter-

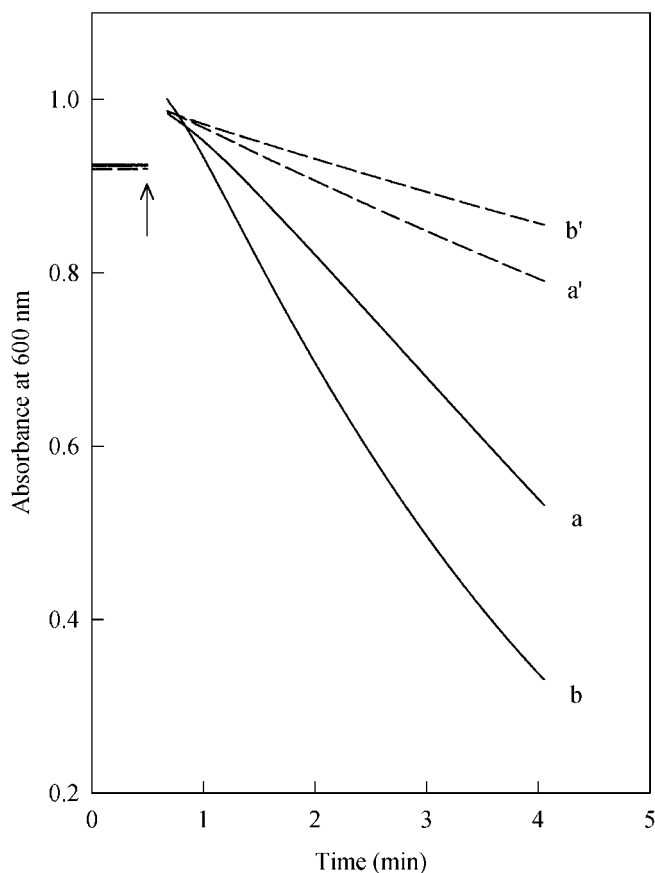


FIG. 7. Isoprimeverose transport in sGDH-containing XylP proteoliposomes with and without entrapped methyl-isoprimeverose. The assays were performed in 500 μ l of 50 mM potassium phosphate, pH 7.0, supplemented with 50 μ M Cl_2Ind and 3.2 mM isoprimeverose (solid lines) or 1.2 mM maltose (dotted lines). The reactions were started (indicated by arrow) by the addition of 5 μ l of XylP proteoliposomes (final concentration: 18 μ g/ml XylP). *a* and *a'*, unloaded proteoliposomes; *b* and *b'*, proteoliposomes loaded with 25 mM methyl-isoprimeverose.

nalized methyl-isoprimeverose on the uptake of isoprimeverose. Methyl-isoprimeverose is not oxidized by sGDH due to the methyl group at the C_1 position of the sugar. When comparing the isoprimeverose oxidation rates of methyl-isoprimeverose loaded with unloaded XylP proteoliposomes, the transport rate, based on two independent experiments, increased from 420 ± 22 to 823 ± 70 nmol $\text{mg}^{-1} \text{min}^{-1}$ (Fig. 7, trace *b* versus trace *a*). It should be noted that, although methyl-isoprimeverose cannot be oxidized by sGDH, the high concentration present in the proteoliposomes somewhat inhibited the sGDH activity as was observed in the control experiments with maltose as substrate (Fig. 7, trace *b'* versus *a'*).

XylP-mediated Uptake Is Stimulated by the Membrane Potential—To test whether XylP-mediated uptake is stimulated by the membrane potential, proteoliposomes prepared in potassium-containing media were diluted into the same buffer (no gradient) or in sodium-containing buffer ($\Delta\psi$). From measurements of the fluorescence of the $\Delta\psi$ -indicator probe diSC₃(5), we could show that 0.25 μ M valinomycin was sufficient to rapidly generate a $\Delta\psi$ and that this potential was stably maintained for several minutes. The $\Delta\psi$ could be dissipated by the addition of 0.1 μ M nigericin (data not shown). As shown in Fig. 8A, transport of isoprimeverose was initially stimulated upon addition of 0.25 μ M K^+ ionophore valinomycin, but the rate of uptake gradually decreased (compare traces *a* and *b*). This inhibitory effect was immediate and even bigger when the proteolipo-

somes were incubated with valinomycin for 30 s prior to the addition of substrate (Fig. 8B, compare traces *a* and *b*). These observations were reproduced in five independent experiments. Since generation of a $\Delta\psi$ provides a driving force for the passive influx of protons, a pH gradient (inside acidic relative to outside) could be formed. Such a reversed pH gradient will inhibit the uptake of a proton motive force-driven system, as it will lower the total driving force for the uptake reaction. To test this hypothesis, the proteoliposomes were prepared and resuspended in a buffer containing 50 mM methylamine. Methylamine can diffuse across the membrane in the dissociated form and becomes protonated in the vesicle lumen, thereby counteracting a drop in internal pH. Indeed, methylamine was capable of relieving the inhibitory effect of the reversed ΔpH that develops when a $\Delta\psi$ (inside negative relative to outside) is generated (Fig. 8, A and B, trace *d*). The presence of 50 mM methylamine did not have any effect on the transport of isoprimeverose in the absence of a $\Delta\psi$ (Fig. 8, A and B, trace *c* versus trace *a*). These results showed that changes in the internal pH were responsible for the inhibitory effects.

DISCUSSION

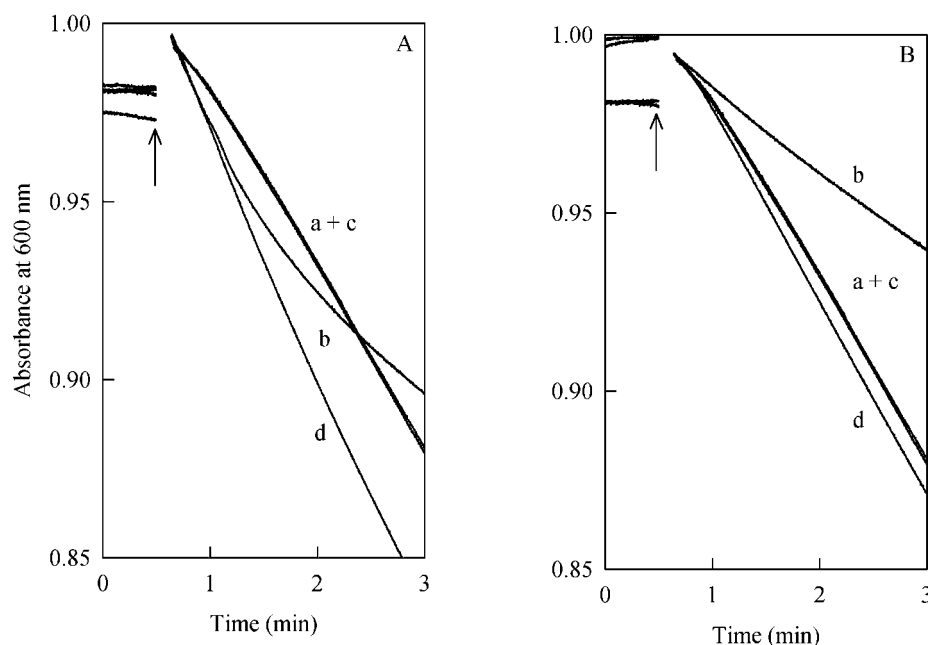
An activity assay system for a membrane transport system requires the incorporation of the protein(s) into lipid membranes as most transporters only catalyze a vectorial translocation reaction and not a conversion of the substrate. Transport reactions are routinely analyzed by following the distribution of isotopically labeled substrates across the membranes, *i.e.* between the outer and inner compartment of a membrane system. Since isotopically labeled xylosides are not available, we devised an alternative transport assay that is generally applicable for the study of carbohydrate reactions. This paper describes the first characterization of an isoprimeverose transporter and a new and more efficient procedure for the synthesis of isoprimeverose and methyl-isoprimeverose.

This new transport assay makes use of a liposomal system with the XylP protein incorporated in the membrane and a PQQ-dependent glucose dehydrogenase (sGDH) enclosed in the liposomal interior. sGDH is readily purified from recombinant *E. coli* cells that overexpress the gene for sGDH (20). In general, the standard error within an experiment is negligible, and even between different experiments the standard error is less than 10%.

The XylP protein of *L. pentosus* has been amplified up to 10% of total membrane protein using the nisin expression system of *L. lactis*. This unique expression system allowed us to grow *L. lactis* to high density without any detectable expression of XylP. Addition of nisin did not slow down the growth and yielded large amounts of XylP within 1 h. In our experience the nisin expression system is superior over any other promoter system for the heterologous (or homologous) overexpression of integral membrane proteins, encoded by genes with a low GC content.

For a detailed characterization of XylP, the protein was purified and functionally reconstituted into proteoliposomes. In view of the fact that highest protein stability was achieved in DDM, a non-ionic detergent with a low critical micelle concentration, the first reconstitution trials were performed by a method based on the insertion of purified protein into preformed, detergent-destabilized liposomes and the use of Biobeads for efficient detergent removal (17, 23, 24). However, proteoliposomes obtained via this method were not active, and freeze-fracture electron microscopic analysis of the membranes showed a very low protein density and a heterogeneous size distribution. Most likely, XylP forms higher oligomeric structures upon the slow detergent removal, which are difficult to insert properly into the liposomes. In order to speed up the reconstitution process, the high critical micelle concentration

FIG. 8. Effects of membrane potential and pH gradient on isoprimeverose transport. The assays were performed in 500 μ l of buffer F or buffer G supplemented with 50 μ M Cl₂Ind. The $\Delta\psi$ was generated by diluting potassium-containing proteoliposomes into these buffers in the presence of 0.25 μ M valinomycin. A, the reactions were started (indicated by arrow) by the dilution of 3 μ l of XylP-containing proteoliposomes into 500 μ l of assay buffer (final protein concentration of 12 μ g/ml), supplemented with 3.2 mM isoprimeverose. B, the reactions were started (indicated by arrow) by the addition of 3.2 mM isoprimeverose to 500 μ l of assay buffer supplemented with 3 μ l of XylP-containing proteoliposomes. Proteoliposomes prepared in buffer D supplemented with 4 mM EDTA were diluted in buffer F (trace a) or in buffer F plus 0.25 μ M valinomycin (trace b); proteoliposomes prepared in buffer E supplemented with 4 mM EDTA were diluted in buffer G (trace c) or in buffer G plus 0.25 μ M valinomycin (trace d).



detergent OG was used. OG can be removed rapidly upon dilution of a protein-detergent-lipid mixture to detergent concentrations below the critical micelle concentration (25). According to Rigaud *et al.* (26), OG is a very efficient detergent in terms of oligomer dissociation and monomer insertion. Although XylP is less stable in OG than in DDM or Triton X-100, the short time course of this method had a positive effect on the reconstitution efficiency. Freeze-fracture electron microscopy studies showed indeed that the particle density in these proteoliposomes is much higher and that the particle size is much more homogeneous.

We could show that XylP is specific for isoprimeverose, and that the carrier does not show any affinity for the monosaccharide xylose. This finding is surprising as the LacS and MelB members of the GPH family do transport galactosides as well as free galactose. Specificity studies with the LacS transporter have indicated that the inward and outward facing binding sites of the protein tolerate large substitutions at the C₁ hydroxyl in both the α - and β -configuration (27).

Kinetic analysis of the transport reaction yielded an affinity constant of 4.5 ± 0.5 mM and a maximal transport rate of 870 ± 45 nmol mg⁻¹ min⁻¹. Preloading the XylP proteoliposomes with the non-oxidizable isoprimeverose analogue methyl-isoprimeverose resulted in a stimulation of the oxidation rate, which is indicative for isoprimeverose/methyl-isoprimeverose exchange transport. Consistent with a proton motive force-driven mechanism, the uptake of isoprimeverose was stimulated by a membrane potential (inside negative relative to outside). Since the membrane potential imposes a force on protons, allowing them to enter the proteoliposomes passively, a pH gradient (inside acidic relative to outside) is anticipated. Such a pH gradient is expected to inhibit a proton motive force-driven uptake mechanism, which was indeed observed for isoprimeverose transport. Strong support for inhibition by a reversed pH gradient came from the experiments in which the membrane potential was generated in the presence of methylamine. This weak base diffuses passively across the membrane, thereby dissipating any pH gradient formed. The uptake of isoprimeverose only displayed the membrane-potential-stimulated phase under these conditions. Although the melibiose transporters (MelB) of the GPH family are stimulated by sodium ions (1), Na⁺ had no effect on the uptake of isopri-

meverose by XylP. We thus conclude that the proton rather than the sodium motive force most likely drives the uptake. Finally, the experiments indicate that effects of a membrane potential or a pH gradient on sugar transport are readily observed with the here presented spectroscopic assay. Since the uptake is not measured via radio-isotope distribution, but via downhill influx of sugars, rates can be measured very accurately. This clearly has advantages in the analysis of mutants in which the transport of sugar and cation (proton) are uncoupled. Such mutants are readily isolated, and they have been instrumental in the understanding of the energetics and kinetics of various secondary membrane transport proteins (28–31).

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